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Proliferation and Osteoblast Differentiation Mice Dental Pulp Stem Cells between Enzyme Digestion and Outgrowth Method

(Proliferasi dan Pembezaan Osteoblas Sel Stem Pulpa Gigi Mencit antara Kaedah Pencernaan Enzim dan Eksplan)

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ABSTRACT

The isolation method for dental pulp stem cells (DPSCs) is still unclear to obtain a conducive environment for DPSCs to proliferate. Enzymatic digestion and outgrowth method are two commonly used methods for DPSCs isolation but are not well characterized in mice DPSCs. This study aimed to compare these isolation methods and differentiation potential of mice DPSCs into bone cells. Dental pulp was extracted from mice's incisors and subjected to isolation either by collagenase 1A or culture of pulp tissue in complete alpha-Modified Eagle Medium (αMEM). Both cells isolated were cultured until passage 4 and subjected to in vitro *proliferation and differentiation analysis. Both cells exhibited fibroblast-liked morphology, but cells isolated by enzyme digestion proliferate faster compare to outgrowth method. After 21 days of osteoblast differentiation, DPSCs isolated from enzyme digestion method showed alkaline phosphatase (ALP) activity slightly different as compared to outgrowth method. In conclusion, there is a significant difference between the cells isolated from enzyme digestion compare to outgrowth method with regard to proliferation and osteoblast differentiation. Thus, it is preferable to isolate by enzyme digestion as it is faster and consistent compared to outgrowth method.*

Keywords: Adherent cells; alkaline phosphatase; in vitro*; mesenchymal stem cells*

ABSTRAK

Kaedah pemencilan bagi sel stem pulpa gigi (DPSCs) masih kurang jelas terutamanya bagi mendapatkan persekitaran yang kondusif bagi DPSCs berproliferasi. Kaedah pencernaan enzim dan eksplan merupakan dua kaedah yang biasa digunakan untuk memencilkan DPSCs namun kurang dicirikan pada DPSCs mencit. Kajian ini bertujuan untuk membandingkan kaedah pemencilan dan potensi perbezaan DPSCs mencit kepada sel tulang. Pulpa gigi diekstrak daripada gigi kacip mencit dan pemencilan sel dilakukan sama ada menggunakan kolagenase 1A atau pengkulturan tisu pulpa pada medium lengkap alpha-modified eagle medium *(αMEM). Kedua-dua sel yang dipencilkan dikulturkan sehingga pasaj 4 dan analisis proliferasi dan pembezaan secara* in vitro *dilakukan. Kedua-dua sel menunjukkan morfologi fibroblas namun sel yang diasingkan melalui pencernaan enzim berproliferasi lebih cepat berbanding dengan kaedah eksplan. Selepas 21 hari pembezaan kepada sel osteoblas, DPSCs yang dipencilkan melalui kaedah pencernaan enzim menunjukkan aktiviti alkali fosfatase (ALP) sedikit berbeza berbanding kaedah eksplan. Kesimpulannya, terdapat perbezaan yang signifikan daripada sel yang dipencilkan melalui kaedah percernaan enzim berbanding eksplan terutamanya daripada segi proliferasi dan pembezaan osteoblas. Oleh itu, adalah lebih baik untuk memencilkan sel melalui kaedah pencernaan enzim kerana ia adalah lebih cepat dan konsisten berbanding dengan kaedah eksplan.*

Kata kunci: Alkali fosfatase; in vitro*; sel melekat; sel stem mesenkima*

INTRODUCTION

Dental pulp stem cells (DPSCs) are mesenchymal stem cells that are capable of differentiating into cells of mesoderm origin such as osteoblasts, chondrocytes, adipocytes, neural and vascular tissue (Barbara et al. 2011; Djouad et al. 2010; Nadig 2009). The soft tissue residing in the inner structure of teeth known as dental pulp is a non-invasive source of adult stem cells. Dental pulp contains mesenchymal stem cells that play a significant role in the process of tissue regeneration such as in bone and teeth structure (Shi et al. 2005). The ability of dental pulp stem cells to undergo self-renewal, multilineage differentiation and the lack of consideration in ethical issues against embryonic stem cells makes dental pulp stem cells as an alternative source of stem cells.

The most studied cell with the capability to show primary osteoblasts behavior *in vitro* is MC3T3-E1 cell lines (Sudo et al. 1983). The advantage of using cell lines MC3T3-E1 is in terms of easy maintenance, an unlimited number of cells and a more stable cell phenotype. MC3T3-E1 cell line derived from mouse calvaria at the early stages of differentiation into osteoblasts. Fundamentally, MC3T3-E1 cell lines were used for testing certain chemicals *in vitro* because naturally, it reacts to the

chemical stimulants produced by the body and capable of proliferating in response to these stimulants (Beck et al. 1998). These chemical stimulants produced by the body are known as ascorbic acid and *β*-glycerophosphate. Both of these materials are essential components in cell differentiation into osteoblast, especially during mineralization.

The selection of the appropriate medium for culturing DPSCs is crucial to ensure that isolated cells capable of retaining the multipotential properties throughout the *in vitro* cultivation. Studies by Lopez-Cazaux et al. (2006) and Nakashima (1991) demonstrate that cell culture medium has a potential to modulate human pulp cells behaviour in term of morphology, proliferation, and differentiation. Usually, DPSCs are cultured in Eagle's basal medium, modified Eagle's medium (MEM), Dulbecco's modified Eagle's medium (DMEM) or alpha modified Eagle's medium (*α*-MEM) with the supplementation of ascorbic acid, β-glycerophosphate and dexamethasone is a prerequisite to observe osteoblast-like differentiation, nodule formation and mineralization. Different ionic concentration such as phosphate and calcium in medium may affect the cellular differentiation of DPSCs (Lopez-Cazaux et al. 2006).

In stem cell research discussion regarding suitable isolation method for DPSCs are still ongoing as researchers aim for a conducive and productive environment to culture DPSCs without compromising the quality of the cells to be transplanted. Two widely applied methods for DPSCs isolation are enzymatic digestion and outgrowth. Enzyme digestion method involved exposure of pulp tissues to enzymes such as collagenase, dispase, trypsin or combination of it which function to break down the extracellular matrix of tissue in a short period to obtain single cell suspensions (de Souza et al. 2015). Outgrowth method meanwhile, involves the culture of dental pulp tissue in complete cell culture medium that causes stem cells in the dental pulp to migrate out through a process called chemotaxis. Outgrowth technique is shown to produce a more homogenous population of cells (Huang et al. 2006). Various studies have reported the success of isolating DPSCs by both methods (de Souza et al. 2015; Hilkens et al. 2013). However, further investigation in determining the quality of DPSCs isolated by these methods are required as it affects the selection of a cost-effective method for cell therapy applications.

In 2010, Schnerch et al. published a report on distinguishing between mice and human pluripotent stem cell regulation: The best laid plans of mice and men and highlighted the importance of accounting for species differences when designing strategies for utilization of the clinical potential of human pluripotent stem cells. Mice are used as a suitable model for stem cells research as it shares many similar biological characteristics with a human. Mice DPSCs also has also been used to further characterize the stem cells because of easy handling, small size, low maintenance cost, and shorter generation time about 10 weeks of life (Akmal et al. 2014).

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy suggest three minimum criteria in determining mesenchymal stem cells which consist of plastic-adherent property, specific surface antigen expression and capability to differentiate into osteoblast, adipocytes and chondroblasts *in vitro* (Dominici et al. 2006). This study aimed to compare mice DPSCs isolated by enzyme digestion using collagenase 1A and outgrowth method as well as their proliferation and differentiation potential into bone cells.

Materials and Methods

COLLECTION OF MICE DENTAL PULP

Healthy mice aged 4-6 weeks were obtained from Animal House of Universiti Kebangsaan Malaysia (UKM). This study was approved by UKM Animal Ethics Committee (Ethics approval number, FD/2016/FARINAWATI/27-JULY/768-JULY-2016-MAY-2017-AR-CAT2). Incisors were extracted from mice's alveolar bone and placed in a sterile tube containing a solution of 1X Phosphate Buffer Saline (PBS) (Gibco, Grand Island, NY, USA) and 1% (v/v) penicillin-streptomycin (Himedia, Mumbai, India). With the aid of a stereomicroscope (Leica EZ4, Germany), the apical part of incisors was cut out using scalpel blade and dental pulp removed using surgical forceps.

ISOLATION WITH ENZYME DIGESTION METHOD

Dental pulp was subjected to enzyme digestion as described by Akmal et al. (2014). The dental pulp that has been successfully extracted were placed in tube contained 0.8 mg/mL collagenase 1A (Sigma, USA) in 1X PBS and incubated at 37°C for an hour with every 15 min vortex to accelerate digestion process. After an hour, the enzymatic reaction was neutralized with the addition of 20% (v/v) Fetal Bovine Serum (FBS) (Gibco, Grand Island, NY, USA). Cells obtained were seeded in complete medium consist of αMEM (Gibco, Grand Island, NY, USA), 20% (v/v) FBS and 1% (v/v) penicillin-streptomycin. Complete medium was changed after culturing for 24 h to remove non-adherent cells followed by every three days until cells reached 80-90% confluency.

ISOLATION WITH OUTGROWTH METHOD

Dental pulp tissue obtained was washed with 1X PBS containing 1% (v/v) penicillin- streptomycin before being minced into smaller fragments using scalpel blade. Each part of dental pulp was cultured in complete medium and incubated at 37°C, 95% (v/v) humidity and 5% (v/v) CO_2. Complete medium was changed every three days. Cells were sub-cultured after reached 80-90% confluency and accutase were used to detach cells from the surface of the plate during the subculturing process.

MICE DPSCS *IN VITRO* PROLIFERATION

Cells from fourth passage were utilized for proliferation analysis. Cells were seeded at 2×10^4 cell/cm^2 in

96-well plate for 21 days of analysis. Cells proliferation was measured at three day interval (Day 0, 3, 6, 9, 12, 15, 18 and 21) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. MTT solution and complete media (1: 9) were added to each well containing cells and incubated at 37°C for 4 h. After incubation, the entire medium was discarded and dimethyl sulfoxide (DMSO) and glycine buffer at pH10.5 were added to each well. The absorbance reading is taken at 570 nm with a reference wavelength of 655 nm using ELISA microplate readers (Bio-Rad, USA). Absorbance values obtained are plotted against day of analysis.

OSTEOBLAST CELLS DIFFERENTIATION

A total of 2×10^4 cells/cm^2 of mice DPSCs-ED and DPSCs-OG were cultured in complete medium in the presence of 50 µg/mL ascorbic acid (Sigma, USA) and 10 mM (w/v) β-glycerophosphate (Sigma, USA) for 21 days. Complete medium without differentiation factors was used as negative control for osteoblast differentiation. The differentiation and complete medium were changed every three days.

ALKALINE PHOSPHATASE (ALP) ACTIVITY DETERMINATION

ALP activity of mice DPSCs isolated by enzyme digestion and outgrowth method was determined by lysing the cells using lysis buffer containing 0.1% Triton X-100 (Sigma, USA) in Tris buffer saline (TBS) cold. The cell lysate was transferred into a tube and freeze- thawed for three times. The cells lysate was incubated in 0.1 M $NaNO_3$- Na_2NO_3 buffer (pH 10.0) (MERCK, Germany) containing 2 mM (v/w) $MgSO_4$ (Sigma, USA) and 6 mM (w/v) *p*-nitrophenyl phosphate (pNPP) (Sigma, USA) for 30 min at 37°C. After incubation, the enzyme-substrate reaction was stopped by the addition of 1.5 M NaOH and the absorbance measurement was taken at a wavelength of 405 nm using a spectrophotometer.

Through this method, ALP expressed by cells catalyzes the hydrolysis pNPP to pNP which has a strong absorbance at 405 nm. ALP enzyme activity is expressed in units (U). One unit is equivalent to 1 µmol *ρ*-nitrophenolate released per minute at 37°C. ALP specific activity obtained by dividing the unit of enzyme activity with the total amount of protein (mg). Therefore, ALP specific activity represented as µmol min^{-1} mg^{-1}. Total protein was determined using the Bradford standard curve (Kruger 2009) in the range of 0.180-0.230 while the concentration of PNP was determined using ALP standard curve in the range of 0.043-1.80.

STATISTICAL ANALYSIS

The results obtained are shown as the mean ± standard deviation (SD) from experiments conducted in triplicate (n=3). Statistical significance was analyzed using Student 's t-test. Proliferation and differentiation assay were repeated independently for triplicate and p values less than 0.05 was considered to be significant.

RESULTS

MORPHOLOGY OF MICE DPSCS

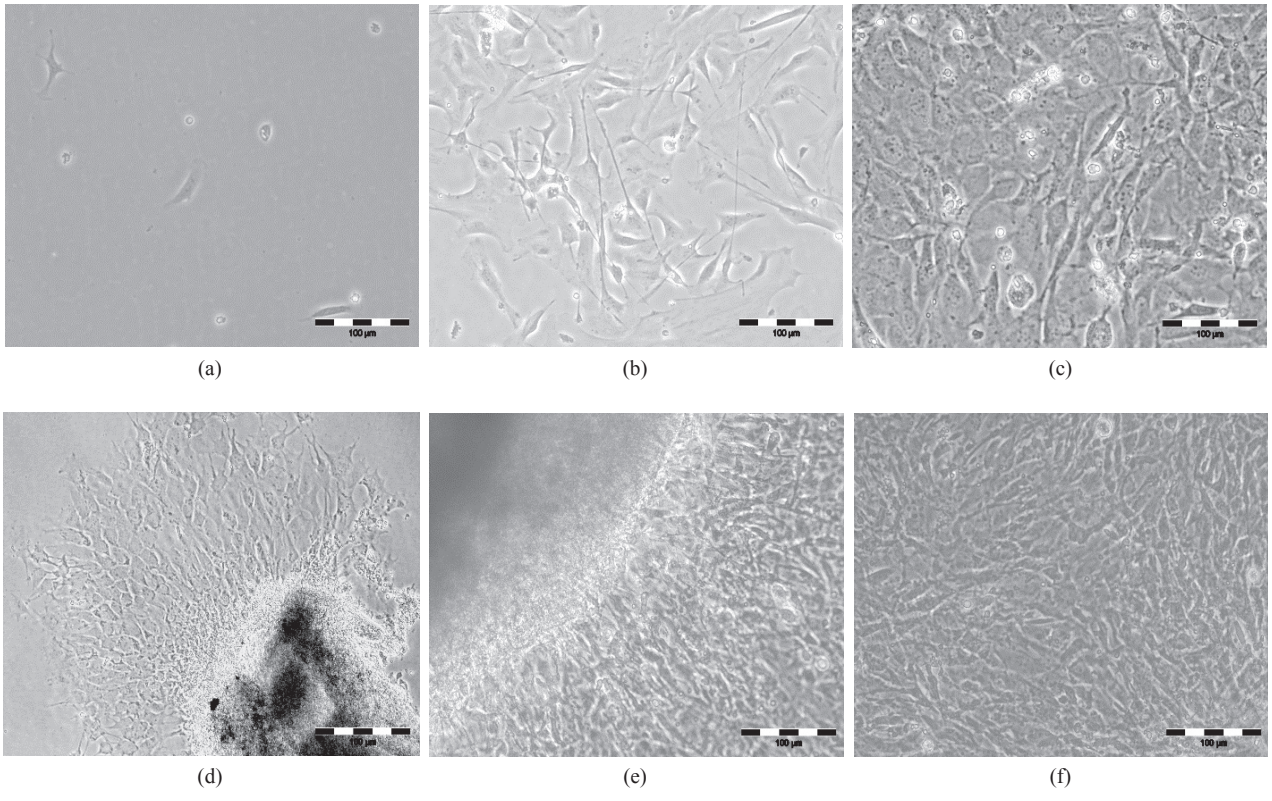
Cells isolated from enzymatic digestion generated adherent cells overnight (Figure 1(a)), whereas the cells obtained through outgrowth method started to proliferate out after 5 days of isolation (Figure 1(b)). Approximately 3 weeks are required for mice DPSCs isolated by enzyme digestion (DPSCs-ED) to reach 70-80% confluency (Figure 1(c)). Meanwhile, mice DPSCs isolated by outgrowth (DPSCs-OG) method required at least 5-6 weeks to migrate from pulp explant and become confluent (Figure 1(d) and 1(f)). At passage 0, the population of DPSCs-ED appears to be more heterogeneous in comparison with the DPSCs-OG. Most cells were spindle-shaped or fibroblast-like, but there were also some cuboidal or polygonal cells being observed. Mice DPSCs-OG method meanwhile, observed to be more homogenous in which most cells exhibited a typical fibroblastic-like shape and were arranged in an orderly fashion (Figure 1(e) and 1(f)). However, with subsequent passages, cells isolated from both methods became more homogenous with appearance and exhibited fibroblast-like morphology (Figure 2).

PROLIFERATION ANALYSIS

Cells viability assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) was performed to access proliferative ability of mice DPSCs isolated by enzyme digestion as well as outgrowth method. Statistical analysis was conducted by t test for comparison of proliferation from day 0 to 21 for both cells isolated between enzyme digestion and outgrowth method (p<0.05, n=3). The proliferation of cells isolated by enzyme digestion was higher compared to outgrowth method. During the observed time frame, a significant difference in proliferation rate was observed in favour of mice DPSCs-ED at day 9 and 21 of plating (Figure 3).

OSTEOBLAST DIFFERENTIATION

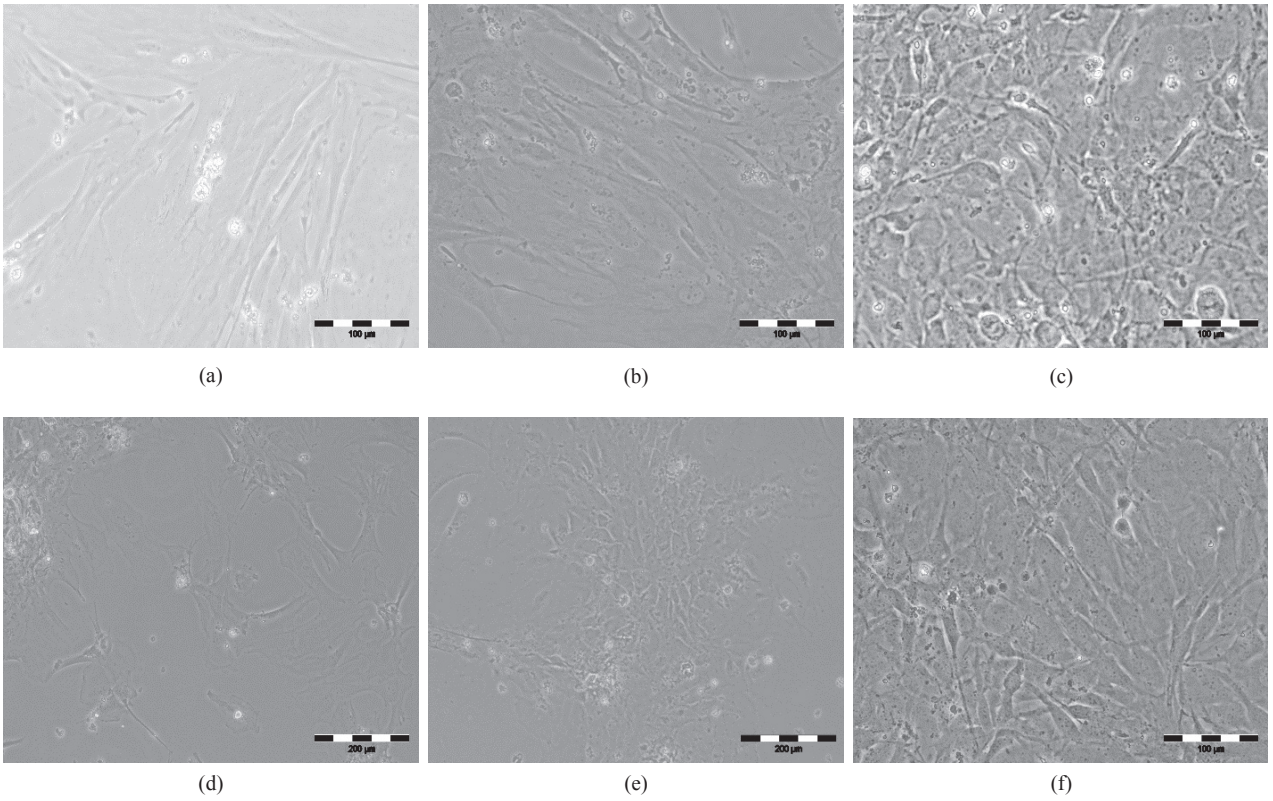
The maintenance of mice DPSCs-ED and DPSCs-OG in osteoblast differentiation medium for 21 days induced production of an enzyme called alkaline phosphatase (ALP). Changes in ALP activity within 21 days of analysis were examined using ALP assay. Comparison of ALP activity between cells with the addition of osteoblast differentiation factor at day 0 (as control) and differentiated mice DPSCs-ED and DPSCs-OG showed slightly different on day 9, 12, 15 and 21. Meanwhile, mice DPSCs isolated by both methods showed a statistically significant difference on day 3, 6, 9, 12, 15, 18 and 21 in favour of differentiated cells. Therefore after 21 days of osteoblast differentiation, mice DPSCs isolated from enzyme digestion method showed ALP activity slightly different as compared to outgrowth method, but no significant difference between the two isolation methods was evident (Figure 4).



(a) (b) (c)

(d) (e) (f)

FIGURE 1. Morphology of mice DPSCs after isolation by enzyme digestion (DPSCs-ED) and outgrowth (DPSCs-OG) method (Magnification 100 µm): (a) Single cell formation at day 1 after isolation (DPSCs-ED); (b) Subconfluent mice DPSCs-ED showed a heterogeneous population of cells after 2 weeks of culture; (c) Confluent mice DPSCs-ED after 3 weeks of isolation; (d) The population of cells migrating out of pulp fragment after 5 days of isolation with (DPSCs-OG); (e) Subconfluent mice DPSCs-OG after 3 weeks of culture; and (f) Confluent mice DPSCs-OG within 5-6 weeks after isolation



(a) (b) (c)

(d) (e) (f)

FIGURE 2. Morphology of mice DPSCs up to their confluency (Magnification 100 µm and 200 µm): (a and e) DPSCs-ED and DPSCs-OG after subculture; (b and e) Sub-confluent in which both cells exhibited fibroblast-like morphology; and (c and f) Confluent mice DPSCs-ED and DPSCs-OG

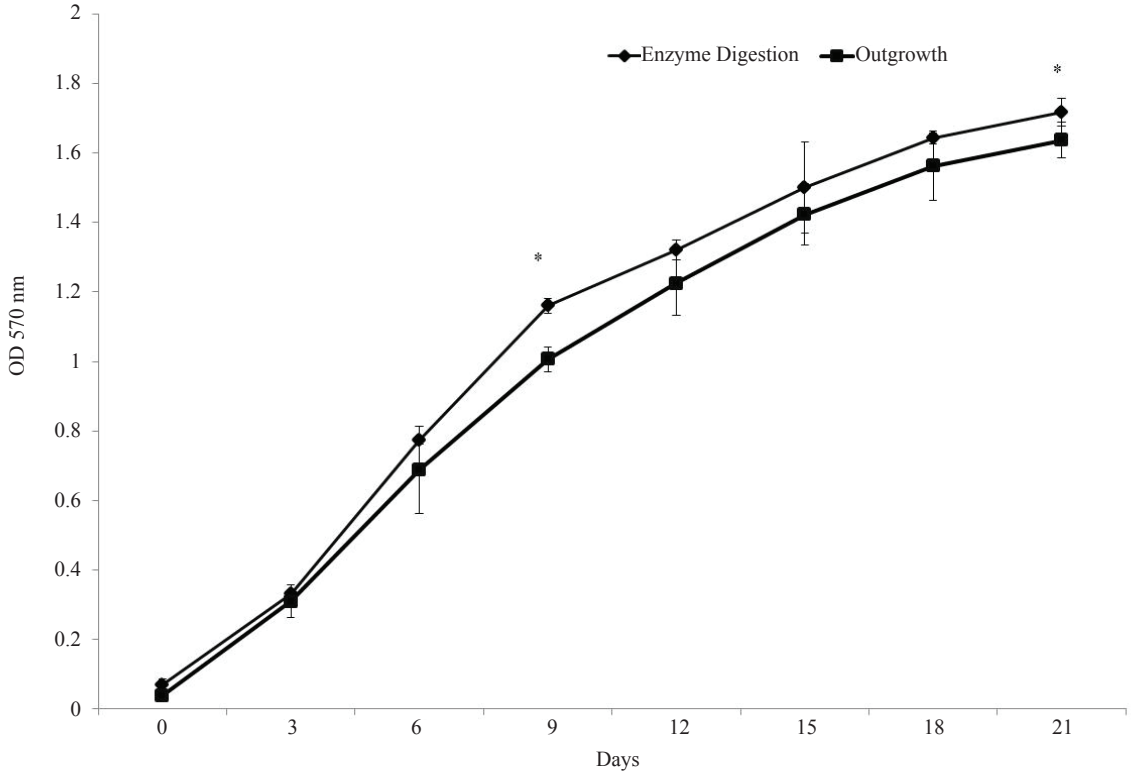


FIGURE 3. Proliferation curve determined by MTT assay. Cells growth curve was applied to the absorbance at 570 nm on an ELISA microplate reader. Cells isolated by enzyme digestion (DPSCs-ED) has higher absorbance values compared to outgrowth method (DPSCs-OG)

All data represent the mean ± SD. Statistical analysis was conducted by t-test for comparison of proliferation from day 0 to 21 between cells isolated by two different methods (p<0.05, n=3). Statistical results demonstrate a significant difference (*) between mice DPSCs-ED and DPSCs-OG at day 9 and 21 (p<0.05, n=3)

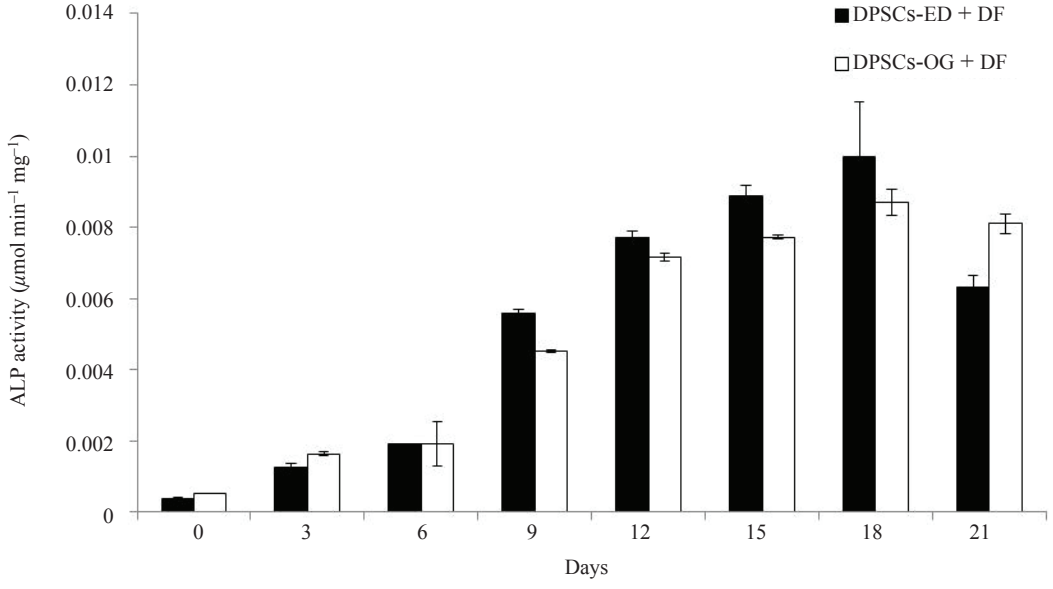


FIGURE 4. Alkaline phosphatase (ALP) profile for mice DPSCs isolated from two different methods. DPSCs-ED + DF, mice DPSCs isolated by enzyme digestion with the addition of differentiation factors; DPSCs-OG + DF, mice DPSCs isolated by outgrowth method with the addition of differentiation factors

Statistical analysis was conducted using a t-test for comparison of ALP activity from day 0 to 21 for cells isolated by two different methods (p<0.05, n=3). Comparison of data between differentiated cells isolated by enzyme digestion and outgrowth method showed a significant difference on day 3, 6, 9, 12, 15, 18 and 21 compared to control (day 0) while no significant different of ALP activity found between these two methods

Discussion

The advancement of stem cell research has led the stem cell community to question the best method to produce numerous functional undamaged mesenchymal stem cells. One major issue is the standard isolation methods for DPSCs as it can affect the differentiation potential of DPSCs. The present study aimed to address several issues regarding isolation method of mice DPSCs and its effect on the quality of cell extracted. Yildirim (2013) suggested two most applied isolation methods for DPSCs, enzyme digestion and outgrowth method. Extracted dental pulp is subjected to digestion by collagenase 1A to obtained single cells suspension or embedded in culture media which supply nutrient to the cells resulting in the cells migrated out of the tissues. Collagenase 1A was used alone to digest pulp tissue as it is the major organic component in dental pulp (Yildirim 2013). Huang et al. (2006) reported the convenience of using outgrowth method during isolation of cells but required more time to allow an adequate number of cells to completely migrate out of tissue nevertheless enzyme digestion is technically challenging with some degree of cells damage but release all cells from tissue. We found that the cellular morphology of mice DPSCs after initial plating was different based on the isolation method applied. Enzymatic digestion seemed to produce a more heterogeneous population of cells with the presence of some cuboidal and polygonal cells. Previously, Bakopoulou et al. (2011) reported an observation of DPSCs derived from human deciduous teeth (SHED) and support earlier reports that enzyme digestion not only allows the isolation of fibroblast-like cells but release of endothelial cells and pericytes. In contrast, outgrowth method yields a more homogeneous population of fibroblast-like cells that migrate out of pulp tissues leaving remaining cells to disintegrate within the tissue (Ellerström et al. 2010; Gronthos et al. 2002; Huang et al. 2006). However, the population of cells becomes uniform in the subsequent passage in which elongated fibroblast-like cells were observed in both cultures. Cells with fibroblast-like morphology observed at passage 4 in this study demonstrate that both isolation methods capable of producing cells with the characterization of mesenchymal stem cells as previously reported by Dominici et al. (2006) and Huang et al. (2006).

Through this study, significant different could be observed on the proliferation ability of mice DPSCs in favor of enzyme digestion method. Cells isolated from outgrowth method proliferate twice slower compared to those obtained by enzyme digestion. A similar observation was supported by a study conducted by Huang et al. (2006) in which both methods were comparatively used for the isolation of DPSCs and were found to give rise to a population of cells at the different rate in which higher in enzyme digestion method. Different in proliferation ability observed is because of enzyme digestion capable of releasing all cells present in the dental pulp during isolation while outgrowth permitting only migrated cells to adhere and proliferate whereas non-migrating cells disintegrate within the tissue (Bakopoulou et al. 2011; Huang et al. 2006). Therefore, it is highly possible that slower proliferation observed during outgrowth method due to only a part of the whole cells population present in the dental pulp migrating out and capable of proliferating during culture.

The osteogenic differentiation of DPSCs-ED and DPSCs-OG had a similar outcome in both cell types. Usually, osteoblast differentiation factors of mesenchymal stem cells derived from bone marrow and dental pulp involve the combination of ascorbic acid and β-glycerophosphate (Huang et al. 2004; Kermani et al. 2014; Seo et al. 2004). Ascorbic acid plays a vital role to maintain and expand mesenchymal differentiation ability as well as reserve proliferation ability without damaging the cells phenotypes, meanwhile *β*-glycerophosphate function as a phosphate donor to induce mineralization within the matrix during formation of osteoblast cells (Kermani et al. 2014). These differentiation factors were used throughout this study to induce osteoblast differentiation in mice DPSCs isolated from both methods. Moreover, mesenchymal stem cells cultured in osteogenic media express marker known as alkaline phosphatase (ALP) which responsible for laying down the mineral and matrix during new bone formation by increasing the local concentration of inorganic phosphate as mineralization promoter and to decrease the concentration of extracellular pyrophosphate which is an inhibitor of mineral formation (Birmingham et al. 2012; Golub & Boesze-Battaglia 2007). In this study, investigation of ALP activity of mice DPSCs-ED and DPSCs-OG showed statistically significantly different ($p<0.05$) on day 3, 6, 9, 12, 15, 18 and 21 between cells under osteoblast induction and the control group. However, no significant difference ($p<0.05$) could be observed in ALP activity between DPSCs-ED and DPSCs-OG, except for an increase of enzyme activity of DPSCs-ED on day 9, 12, 15 and 18 as compared to DPSCs-OG. Studies by Raouf and Seth (2002) and Thomas et al. (2002) shown that osteogenic differentiation stimulates ALP expression occurring between 11 and 25 day meanwhile Nourbakhsh et al. (2008) who study on osteoblast differentiation of SHED implied that ALP activity increased after three weeks of stimulation. These results are consistent with those of previous studies that mice DPSCs establishes via enzyme digestion, and outgrowth method can be induced to differentiate into cells of osteoblastic-type within 21 days of analysis. Even though significant differences ($p<0.05$) in ALP activity between these two methods could be observed on certain days only, enzyme digestion promotes a better potential for cells to differentiate into osteoblast compared to outgrowth method.

Conclusion

Overall, this study found that cells isolated from both methods showed a capability to proliferate and differentiate into osteoblast under appropriate induction. There is a significant difference between the cells isolated from enzyme digestion compare to outgrowth

method with regard to proliferation and osteoblast differentiation which is mice DPSCs isolated by enzyme digestion show high proliferation ability and ALP activity when induce to differentiate into osteoblast. The results of this study suggest that it is preferable to use enzyme digestion during isolation of mice DPSCs as it is faster, consistent and predictable compared to outgrowth method.

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